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Cytochrome *c*-551 of the thermophilic bacterium PS3, DNA sequence and analysis of the mature cytochrome

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The structural gene for cytochrome *c*-551 was isolated from genomic DNA of the thermophilic bacterium PS3. The amino acid sequence of cytochrome *c*-551 as deduced from the DNA sequence consists of 111 amino acid residues and contains one heme *c*-binding site (-CASCH-) located approximately in the middle of the polypeptide. The N-terminus of isolated cytochrome *c*-551 was blocked, but treatment with *Rhizopus* lipase and molecular weight measurement of the mature and lipase-treated forms by ion spray mass spectroscopy suggest that the mature *c*-551 may have 93 or 94 amino acid residues with a diacylated glycerol-cysteine at the N-terminal region. The first 17 or 18 amino acid residues in the N-terminal region of the nascent polypeptide, rich in hydrophobic and basic amino acid residues, may be a signal peptide to translocate the major portion of cytochrome *c*-551 to the extracellular surface and to be processed. Similarity of amino acid sequence of this protein is discussed in relation to other *c*-type cytochromes of bacilli as well as bacterial small cytochromes *c* such as *Pseudomonas aeruginosa* cytochrome *c*-551 and cytochrome c_6 of cyanobacteria.

Introduction

Not much is known about the *c*-type cytochromes of Gram-positive bacteria, in contrast to the information about the cytochromes *c* in Gram-negative bacteria, some of which are rather closely related to the mitochondrial cytochrome *c*. We have been working on the respiratory chain of the thermophilic bacterium PS3 as a model for a typical Gram-positive bacterial system. There are at least three *c*-type cytochromes in PS3: One is present as a component of subunit II of the cytochrome caa_3-type oxidase [1]. This cytochrome *c* constitutes the C-terminal part of the subunit II polypeptide [2,3]. Another *c*-type cytochrome (cytochrome *f* or c_1) is found in the cytochrome b_6f (bc_1) complex showing an asymmetrical alpha peak at 554 nm [4]. There is no indication that the third *c*-type cytochrome is operating between cytochrome b_6f complex and cytochrome oxidase (caa_3), since these form a supercomplex in PS3 [5], and it was observed that the addition of yeast cytochrome *c* did not accelerate quinol oxidase activity. However, PS3 cells, cultured under air-limited conditions, produced one more *c*-type cytochrome, named cytochrome *c*-551 [6,7] with a concomitant change of cytochrome pattern, including the terminal oxidases [8,9]. Cytochrome *c*-551 was extracted with a mild surfactant, cholate, and purified as a 10 kDa protein [7]. The cytochrome seemed to mediate electron flow from the cytochrome b_6f complex to an alternative terminal oxidase [7,9].

In Gram-positive bacteria other than PS3, a few small-sized cytochromes *c* have been isolated without elucidation of their specific function [10–15]. In addition, Von Wachenfeldt and Hederstedt recently cloned the gene (*cccA*) encoding a 13 kDa cytochrome *c*-550 of *Bacillus subtilis* and succeeded in over-expressing it in *B. subtilis* and *Escherichia coli* [15,16]. They also deleted the gene, but the deletion mutant survived as before [15]. Here we report the gene structure and the deduced amino acid sequence of PS3 cytochrome *c*-551 as well as data suggesting post-translational processing at the N-terminus.

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The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number x63125.

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Abbreviations: PCR, polymerase chain reaction; ORF, open reading frame; bp, base pair; PTH, 2-phenyl-3-thiohydantoin derivative.

Experimental procedures

Materials. T_4-DNA ligase, the DNA polymerase of *Thermus aquaticus* (*Taq* polymerase), restriction enzymes, plasmid vectors pUC18 and pUC19, and M13 bacteriophages mp18 and mp19 were obtained from Takara Shuzo Co. (Kyoto, Japan). [α-^{32}P]dCTP and Hybond-N + (nylon membranes for DNA blotting) were purchased from Amersham Corp. Lipase from *Rhizopus delemer*, fine grade, chymotrypsin (sequencing grade), and Butyl-Toyopearl (Butyl-Fractgel) were products of Seikagaku Kogyo Co. (Tokyo), Boehringer (Mannheim) and Tosoh Co.(Tokyo), respectively.

Purification of cytochrome c-551. The procedure was as described previously [9], except that the following hydrophobic chromatography step was introduced between the original steps 1 and 2: To the sample from step 1, ammonium sulfate was added to 60% saturation, and then the mixture was centrifuged at 20 000 × *g* for 15 min. The supernatant fraction was applied on a Butyl-Toyopearl column (2 × 10 cm). The red band was eluted by adding 0.2 M ammonium sulfate containing 10 mM Tris-HCl buffer (pH 8.0).

Chemical and enzymic cleavages and peptide purification. Native PS3 cytochrome *c*-551 (30 nmol) was cleaved with cyanogen bromide (30 μmol) in 50% trifluoroacetic acid at 25°C for 24 h. Two peptides, one without heme and one with heme, were separated on a C_{18} column (Lichrosorb RP-18, Cica-Merk, Tokyo) by reverse-phase HPLC with a liquid chromatograph (Waters M45). The mobile phase was a mixture of acetonitrile containing 0.05% trifluoroacetic acid and 0.05% trifluoroacetic acid at a ratio varying from 0 : 100 to 95 : 5. The flow rate was 1 ml/min. Because of limitation of the column size, this chromatography was repeated five times, and the fractions containing heme *c* (1–2 μmol) were pooled, and concentrated in vacuo. A part of this heme peptide resulting from cyanogen bromide cleavage was then treated with 2-nitrophenylsulfenyl chloride (100 μmol) in 66% acetic acid for 10 min at room temperature to remove heme *c* [17]. The cyanogen bromide peptide containing heme *c* (200–400 nmol) was treated with chymotrypsin (5 μg) in 0.2 M Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ at 37°C for 2.5–5 h. The chymotrypsin-digested peptides were fractionated by reverse-phase HPLC.

Peptide sequencing. Sequential degradation from the NH_2-terminus of the peptides and analysis of phenylthiohydantoins were carried out with a gas-phase Sequenator (Applied Biosystems, model 470A) equipped with a model 120A phenylthiohydantoin-amino acid analyzer.

Molecular cloning and DNA sequencing. The methods used for molecular cloning were based on those of Maniatis et al. [18]. Oligonucleotides used for PCR were synthesized using an Applied Biosystems model 80B DNA synthesizer. PCR was performed according to the manufacturer's protocol with PS3 DNA (1 ng) as template in a thermal cycler (Perkin-Elmer Cetus). A combination of 94°C/45°C/72°C (1 min each) was repeated 25 times. The products of the PCR reaction were centrifuged in the cup of a Centricon (Amicon. Beverly, MA) to remove primers and mononucleotides, dephosphorylated by calf intestine alkaline phosphatase (2 μg), and ligated to the M13 mp19 vector cleaved with *Sma*I.

The nucleotide sequence was determined by the Sanger dideoxy chain terminator method [19] using an

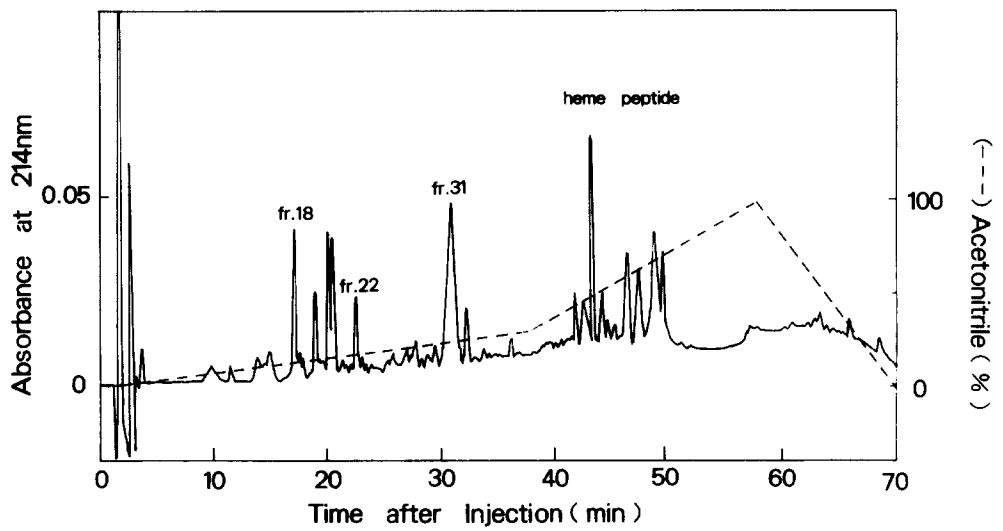

Fig. 1. Separation of fragments obtained by chymotrypsin treatment of cyanogen bromide-treated PS3 cytochrome *c*-551. The heme *c*-containing fragment (20 μg) obtained after cyanogen bromide treatment of PS3 cytochrome *c*-551 (Experimental procedures) was incubated in 0.2 M Tris chloride buffer (pH 8.0) containing 1 mM $CaCl_2$ and chymotrypsin (1 μg) at 37°C for 5 h. A reverse-phase HPLC elution profile of the digested fragment is shown.

Applied Biosystems model 370A DNA sequencer, and fluorescent universal primers (No. 400386, Applied Biosystems, CA). The chain elongation reaction was carried out with Taq polymerase at 60°C. The unique restriction sites for *Spl*I and *Nae*I (Fig. 4) were used for the subcloning, and the DNA sequences obtained covered all regions in both directions. *E. coli* JM105 and HB101 strains were used as the hosts of M13 and pUC18/19, respectively.

Lipase treatment. Effects of lipase-treatment were analyzed by reverse-phase HPLC on a C_4 column (Waters microbondasphere, 5 μ), and by ion spray mass spectrography. The mass spectra were measured in a JMS-LX2000 mass analyzer of the Applied Research Center of Nippon Denshi Co. with an electron ionization mode. The accelerating voltage was 2.0 kV.

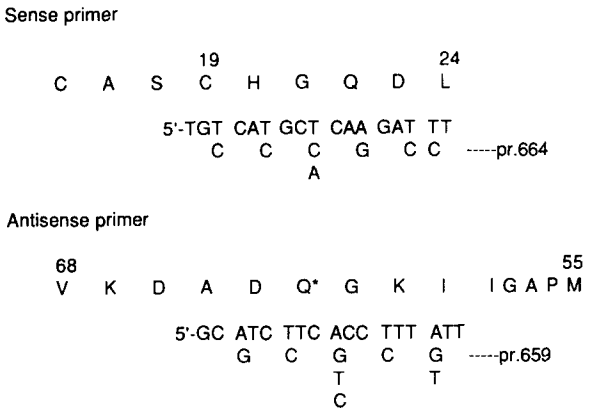

Fig. 2. Design of primers for cloning using PCR. Q* determined by peptide sequence analysis was not correct, but the primer (pr. 659) worked well.

Results

Partial protein sequencing

The N-terminus of cytochrome *c*-551 seems to be blocked, since the yield of PTH-amino acids with the gas-phase peptide sequencer was below 5% of the amount expected up to at least 5 cycles. The block does not seem to be a formyl group, since treatment of the polypeptide with 1 M HCl in methanol [20] did not help. Cyanogen bromide cleavage of the native cytochrome gave two peptides that were separated. Amino acid sequence analysis of the smaller one, without heme *c*, indicated that its sequence was -PAGI-IKGQDADKVAEWLAKK. The larger fragment with heme *c* gave a very small amount of PTH-amino acids, and was therefore treated with chymotrypsin. Fig. 1 shows the reverse-phase HPLC elution pattern. Fractions 18 and 22 gave the following two sequences, respectively: SKDEIKNI- and SGGVGPNL-. In contrast, fraction 31 yielded hardly any PTH-amino acids. The peptides recovered between the fractions 41–50, contained heme *c* as judged by the absorbance at 408 nm. One of the peaks (fraction 43) had the sequence KQNXASXHGQ-; and the 7th cycle gave a pink fraction, indicating that a derivative of heme *c* was recovered here. The chymotrypsin digestion of the heme peptide derived from cyanogen bromide cleavage deprived of heme *c* by 2-nitrophenyl sulfenyl treatment [17] was fractionated by reverse-phase HPLC. One of the resultant fractions had the sequence KQNXASX-HGQDL-.

Cloning of the gene for cytochrome c-551

We prepared two sets of oligonucleotides targeted to the DNA sequences corresponding to the heme *c*-binding region and the C-terminal region, respectively, as shown in Fig. 2. PCR was carried out on PS3 chromosomal DNA using these oligonucleotides as a set of primers. Acrylamide gel electrophoresis indicated products of about 120 bp and 300 bp. The 120 bp DNA was recovered from the gel and ligated into *Sma*I-cleaved M13 vector mp19. Nucleotide sequencing of five resulting clones revealed one clone (clone 3) containing the primer sites.

Clone 3 was used as a probe to clone the structural gene for cytochrome *c*-551. Fig. 3 shows a typical result of Southern blotting. The 3.5 kbp *Eco*RI fragment to which the probe hybridized was cleaved by both *Sal*I and *Pst*I, and the size of the fragments resulting from *Sal*I or *Pst*I cleavage was about 1.2 kbp. These restriction patterns were exploited to clone the gene for cytochrome *c*-551; 3.5 kbp DNA fragments obtained by *Eco*RI cleavage were recovered from the agarose gel, cleaved by both *Sal*I and *Pst*I, and then ligated to *Sal*I- and *Pst*I-cleaved pUC19 vector without fractionation. *E. coli* was transformed with the ligated vector. Plasmid was prepared from 60 "white" colonies. One plasmid hybridized well with the probe prepared by PCR with prs. 664 and 659 as the primers and clone 3

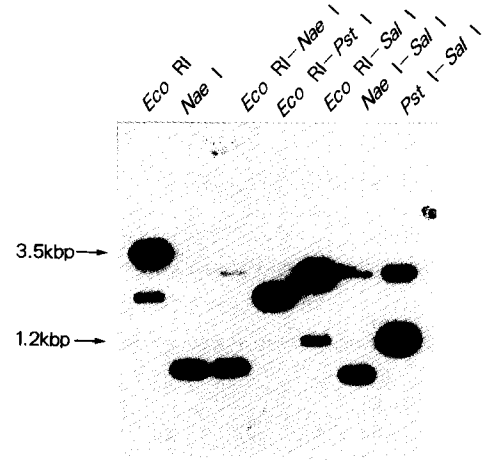

Fig. 3. Southern blot analysis of PS3 chromosomal DNA hybridized with clone 3 as the probe. The hybridization was carried out at 60°C for 3 h in the rapid hybridization buffer (Amersham).

as template. The DNA of this pUC19 clone contained the structural gene for PS3 cytochrome *c*-551.

DNA and deduced protein sequences

Fig. 4 shows the nucleotide sequence of the *Sal* I-*Pst* I fragment (1192 bp) containing the gene (*cccA*, named after the *B. subtilis* cytochrome *c*-550 gene) for cytochrome *c*-551. The open reading frame for *cccA* consists of 333 bases; it starts with an ATG at position 518 and terminates with a TAA at position 852. There is a possible transcriptional terminator after the translational stop codon as indicated. There is no clear -35/-10 structure for a promoter in the sequence upstream of the ATG start codon. The ORF preceding *cccA* has not yet been identified, but the gene is not homologous to *rpoD* (*sigA*) [21] found in the case of *B. subtilis* [15], the *E. coli cyoA-E* operon [22], or the *E. coli cyd* operon [23].

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Sal I
GTCGACCGGCGGCACCGATTTGGCAGCGCAAATCATCCATAAATATACCGGGCTGTCGCT   60
 S  T  G  G  T  D  L  A  A  Q  I  I  H  K  Y  T  G  L  S  L

TGGCATGTGCGTCATTTTGATCGATGGCTTGATCGTGTTGACCGCGGCGTTTGTGTTTGA  120
 G  M  C  V  I  L  I  D  G  L  I  V  L  T  A  A  F  V  F  D

TATTGAACGGGCGCTCTATGCGTTGATCGCGTTGTATGTGACGAGCAAAACGATCGACCT  180
 I  E  R  A  L  Y  A  L  I  A  L  Y  V  T  S  K  T  I  D  L

CGTCCAAGTCGGGCTTGGGTATTCGAAAATCGCTCTTATTATTACGAATGAAGAAGAAAA  240
 V  Q  V  G  L  G  Y  S  K  I  A  L  I  I  T  N  E  E  E  K

AGTGCGCCGCGCCATTTTGCACGAAATCGACCGCGGAGTGACGCGCTTGCCCGCGTATGG  300
 V  R  R  A  I  L  H  E  I  D  R  G  V  T  R  L  P  A  Y  G

CGGTTACACGGAACATGAGCGGCCGGTGTTGATGTGCGTCGTCGCGCAATCGGAGTTCAC  360
 G  Y  T  E  H  E  R  P  V  L  M  C  V  V  A  Q  S  E  F  T

GAAATTGAAACAATTGGTCCGAACCATTGATCCGACAGCGTTTGTCATCGTTGCGAACGC  420
 K  L  K  Q  L  V  R  T  I  D  P  T  A  F  V  I  V  A  N  A

                    Spe I
CGCTGAGGTACTCGGCGAAGGATTTCAACGTACGTAAAACGGAAGAAATCGGTTATAATA  480
 A  E  V  L  G  E  G  F  Q  R  T  *

                S.D.                    518
AATGAGAACGAAATTTTTGTTTTCAGGGGGAGTTCGCCATGAAATGGAAATTGGCTGCGA  540
                                 M  K  W  K  L  A  A  M          8
TGTTCCTTGGCGTTTCGCTCGCGCTTGCCGCGTGCGGCGGTGGCGGGGACAATGCCGGGG  600
 F  L  G  V  S  L  A  L  A  A  C  G  G  G  G  D  N  A  G  E   28

AGAAAAACGGCGGCAGCAACGGCGGAGGGGATACAGCAGCGGCTGCCGAGCAAATTTTTA  660
 K  N  G  G  S  N  G  G  G  D  T  A  A  A  A  E  Q  I  F  K   48

AACAAAACTGTGCGTCCTGTCATGGACAAGACCTGTCGGGCGGGGTCGGCCCGAACTTGC  720
 Q  N  C  A  S  C  H  G  Q  D  L  S  G  G  V  G  P  N  L  Q   68

AAAAGGTTGGAAGCAAGTATTCCAAAGATGAAATTAAAAACATTATCGCCAACGGCCGCG  780
 K  V  G  S  K  Y  S  K  D  E  I  K  N  I  I  A  N  G  R  G   88
    Nae I
GCGCGATGCCGGCAGGAATCATTAAAGGGGAAGACGCCGACAAAGTGGCGGAATGGCTGG  840
 A  M  P  A  G  I  I  K  G  E  D  A  D  K  V  A  E  W  L  A  108

CTGCGAAAAAATAATGGACGGATGTTTCTGACTCTTTGAAAGGAAAAGATCGTTTCATTC  900
 A  K  K  *                                                    111

AAGGAAGGTGCACCGCCTTGTTCGCTTAAGAACGGGGCGGTGTTTTCTGTTTTTGCATCT  960

TTTGTCCTAGTTTCTCCATTGTTCCCCATTTGAAATATAAATGTAACTGTAGATGAGCAA 1020

ATTCGCTAACATAAATTTACAACCAAACAAAAAAAGAGACATGAACCCGATTCCTTGATT 1080

AGAATAGATGTGTCACCAAAACTATTCACAAGGAGGTTCATGTCTCATGAATAGATTAGC 1140
                                                    Pst I
ACATCATCAAGGAATCCACAAGTTTTTCTTCACGCTGGGGTTGACGCTGCAG  (1192)
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Fig. 4. DNA sequence of *cccA* and amino acid sequence of PS3 cytochrome *c*-551. The underlined amino acid residues indicate the sequences determined from the peptide. The putative Shine-Dalgarno sequence is boxed, and the arrows show an inverted repeat putatively being a transcription terminator of the gene. The amino acid sequence for an open reading frame present upstream of the structural gene for cytochrome *c*-551 is also translated.

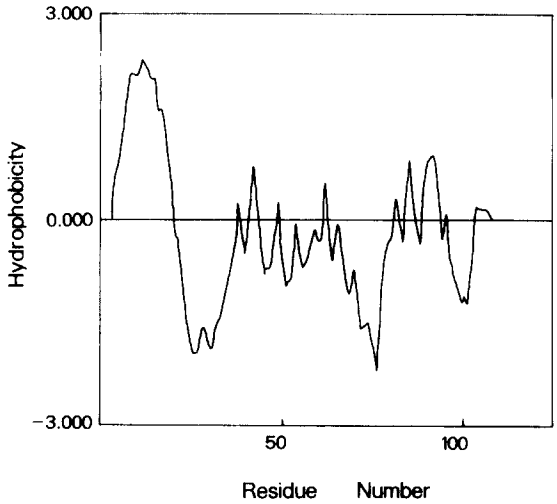

Fig. 5. Hydropathy profile of the PS3 *cccA* gene product. The procedure of Kyte and Doolittle [30] was used for calculation with a window of 8 residues.

The deduced amino acid sequence in this open reading frame is shown in Fig. 4; PS3 cytochrome *c*-551 is probably produced as a 111 amino acid residue protein containing one heme-*c*-binding site (-CASCH-) in the middle. The N-terminal region of this sequence

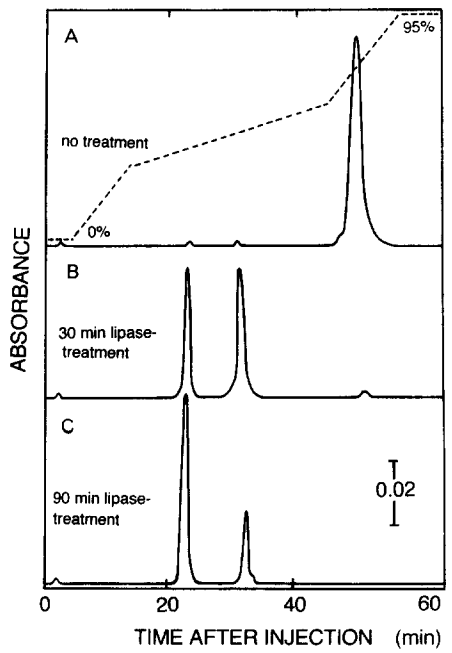

Fig. 6. Elution profile of PS3 cytochrome *c*-551 in C_4 reverse-phase chromatography and effects of lipase-treatment on the profile. The PS3 cytochrome (100 nmol) was treated with *Rhizopus* lipase (20 μg) in 1.5 ml of 20 mM Tris chloride buffer (pH 8.0) at 36°C (B and C). Aliquots (40 μl) of the solution, before (A) and after the treatment were analyzed on the C_4 column by HPLC. The solvent was water and acetonitrile, both containing trifluoroacetic acid (0.03%), and a gradient elution was done as shown by the dotted line. Absorbance was monitored at 400 nm. The flow rate was 0.7 ml/min.

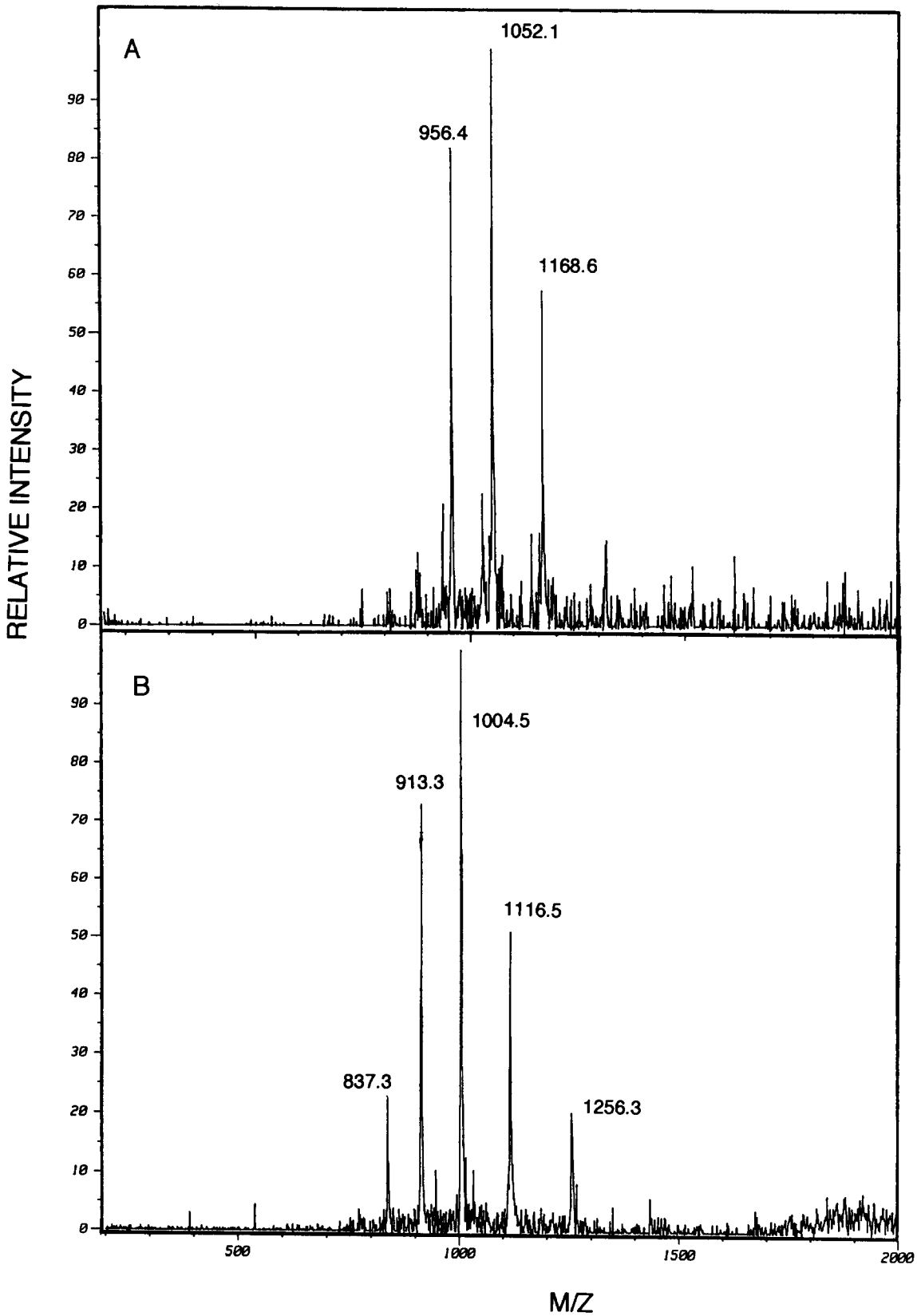

Fig. 7. Ion spray mass spectra of mature PS3 cytochrome *c*-551 (A) and of the lipase-treated cytochrome (B). The 52 min fraction of Fig. 6A, and the 22 min fraction of Fig. 6C were used. The molecular weight (M) was calculated by the following equation; $M = n(N_n - 1)$, where N_n is m/z due to the multi-charge (n) ion, and n can be calculated as follows; $n = (N_{n+1} - 1)/(N_n - N_{n+1})$.

is rich in both hydrophobic and basic amino acid residues, showing the characteristics of a signal peptide. In fact, the molecular mass of mature PS3 cytochrome *c*-551 was determined to be 10 kDa [7], and its N-terminus is blocked (the present work). Thus, the nascent cytochrome *c*-551 is likely to be transferred to the outside of the cellular membrane, modified at this site, and to work there as a peripheral membrane-protein. However, the deduced amino acid sequence of PS3 cytochrome *c*-551 has no hydrophobic region except the putative N-terminal signal sequence (Fig. 5). Thus, we assume that the mature protein may be a lipoprotein with fatty acids, as in the case of the cytochrome subunit of photosynthetic reaction center of *Rhodobacter viridis* with a diacylated glycerol-residue attached to the N-terminal cysteine residue via a thioether bridge [24]. In fact, PS3 cytochrome *c*-551 showed very hydrophobic elution profile by reverse-phase chromatography, as shown in the next section.

Effects of lipase-treatment on the molecular mass

Fig. 6 shows elution profiles of PS3 cytochrome *c*-551 by reverse-phase chromatography. The cytochrome eluted at 52 min (A), while the cytochrome treated with *Rhizopus* lipase showed new peaks at 31 min and 22 min (B and C). The original 52 min and the derived 22 min fractions were analyzed by ion spray mass spectroscopy as shown in Fig 7. The molecular mass of native cytochrome *c*-551 was calculated to be 10509.5 (average of $z = 9–11$), while that of the cytochrome eluted at 22 min was 10037.7 (average of $z = 8–12$). The difference, 471.8, seems to correspond to the two fatty acyl side chains. The presence of two fatty acyl residues is also supported by the appearance of the third peak at 31 min, when the incubation time of lipase-treatment was not long enough (Fig. 6B). The peak at 31 min is probably due to mono-acyl derivative of mature cytochrome *c*-551. Even after the lipase treatment N-terminal of cytochrome *c*-551 was blocked.

Discussion

The present DNA and protein sequence study on an unique cytochrome *c* from a thermophilic *Bacillus* PS3 showed that the *cccA* gene product for the cytochrome is composed of 111 amino acid residues, and should be processed into the 10 kDa mature lipoprotein with a blocked N-terminus. The mature cytochrome subunit of *R. viridis* reaction center was reported to have a diacylated Cys-residue with an amino group at the N-terminus [24]. The lipase treatment and molecular mass determination (Figs. 6 and 7) seem to support the idea that the new N-terminus of mature cytochrome *c*-551 is Cys-19 with a diacylated glycerol residue. The total mass of amino acid residues 19 to 111 (Fig. 5), protoheme (equivalent of heme *c*), and glycerol-residues is 9924.5 = 9234 + 616.5 + 75 − 1, a little smaller than the molecular mass of the lipase-treated cytochrome at 22 min. The difference between 10037.7 and 9924.5 (113.2) should be due to other modification(s), including the N-terminal blocking. If an acetyl group blocks the N-terminus, there is a possibility that the signal peptidase cut the peptide bond between Ala-17 and Ala-18, since the mass of acetyl-alanyl group is 114, and bacterial type I signal peptidase is known to processed after alanine residues [25]. However, the fact that all known lipoproteins contain a modified N-terminal cysteine [24] is strong enough to speculate that the N-terminus of cytochrome *c*-551 is also the modified cysteine. It is also possible some minor modification(s) such as methylation may occur in the cytochrome, if the blocking group of the N-terminus is an acetyl group, so that the molecular weight is to be about 10509. Further work is necessary to determine the precise structure of this cytochrome. We are constructing an over-expression system in a thermophilic *Bacillus* strain.

Von Wachenfeldt and Hederstedt [15,16] recently cloned the gene (*cccA*) for *B. subtilis* cytochrome *c*-550 which is clearly homologous with PS3 cytochrome *c*-551. The *B. subtilis cccA* gene encodes a 120 amino-acid-residue polypeptide, and the mature cytochrome *c*-550 was 13 kDa with initiating methionine residue and a sufficiently hydrophobic signal sequence at the N-terminal region. This signal peptide may anchor the cytochrome to the membrane. If so, PS3 cytochrome *c*-551 and *B. subtilis* cytochrome *c*-550 become membrane-bound in quite different ways. Anyway, it seems important for cytochrome *c* of Gram-positive bacteria to be membrane-bound. In the case of cytochrome *c*, which is the substrate of cytochrome aa_3-type oxidase, it is integrated as a part of subunit II of the oxidase itself [3,26].

Cytochromes *c* are classified into several groups [27,28], but the protein sequence of cytochrome *c* from Gram-positive bacteria has not been reported until very recently. Fig. 8 shows the alignment of PS3 cytochrome *c*-551, *B. licheniformis* and *B. subtilis* cytochrome *c*-550 with those of *Pseudomonas aeruginosa* *c*-551 (c_8 group) and *Anacystis nidulans* cytochrome c_6 (c_6 group), cytochromes c_8 from Gram-negative bacteria such as denitrifiers, and cytochromes c_6 from cyanobacteria and algal chloroplasts are known to be small *c*-type cytochromes having one heme *c* in the N-terminal region. The three *Bacillus* cytochromes *c* clearly have common amino acid residues with both c_8 type and c_6 type, and also clearly different from both types. The *Bacillus* cytochromes *c* show a deletion apparently at the five positions. Two sets of deletions are common with the c_8 group, and the other two sets with the c_6 group. It is thus likely that the small *Bacillus* cytochromes *c* are most similar to the small

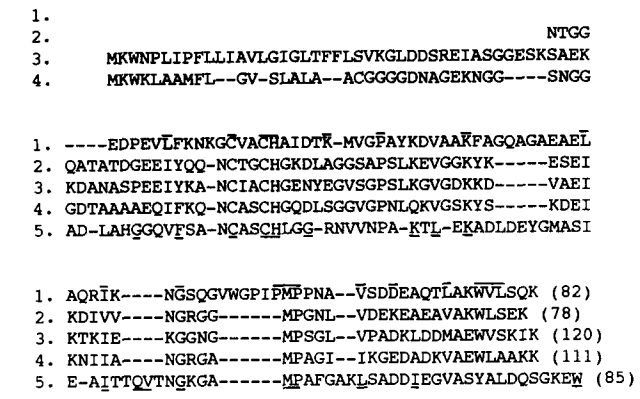

Fig. 8. Alignment of small sized bacterial cytochromes *c*. The line on the top of 1, and the underline of 5 indicate that the residues are consensus among the c_8-type and c_6-type cytochromes *c*, respectively. 1; *Pseudomonas aeruginosa* c-551 [31]. 2; *B. licheniformis* (Van Beeumen J., personal communication as reported in Ref. 14). 3; *B. subtilis* c-550 [15]. 4; PS3 c-551 (present work). 5; *Anacystis nidulans* c_6 [32].

cytochrome *c* group with c_8 and c_6, but are different from both c_8 and c_6. This situation seems to be in accordance with the notion that Gram-positive bacteria are more closely related to cyanobacteria, as concluded by comparison of 16S rRNA sequences by Woese et al. [29]. We have already reported that menaquinol-cytochrome *c* reductase of *Bacillus* PS3 is more similar to cyanobacterial cytochrome b_6f complex than to purple bacterial or mitochondrial cytochrome bc_1 complex [4].

PS3 cytochrome *c*-551, produced abundantly when cells were cultured under air-limited conditions, donates electrons to an alternative terminal oxidase other than cytochrome caa_3-type [1,9]. Since PS3 cytochrome b_6f complex and cytochrome caa_3-type oxidase are working when the cells are cultured with vigorous aeration as reported previously [5], the thermophilic *Bacillus* PS3 has at least two sets of terminal oxidases [9]. It is interesting that the alternative oxidase, adapted to lower oxygen concentration but less efficient (not proton-pumping), uses cytochrome *c*-551 which is related to cytochrome c_6. Since *B. subtilis* is known to have 13 kDa cytochrome *c*-550 [15] and cytochrome caa_3-type oxidase [26], it may be general in *Bacillus* to have two sets of oxidases with two different *c*-type cytochromes for adapting to different growth conditions.

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